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14. ABSTRACT In the grant proposal we have hypothesized that tumor-derived bone morphogenetic protein-6 (BMP-6) induces tumor associated macrophages (TAMs) to express interleukin-6 (IL-6) via a crosstalk between the Smad-dependent and the p38 pathway; IL-6 in turn drives neuroendocrine (NE) differentiation of prostate cancer cells. To test this proposal, three specific aims were proposed: 1) To investigate the mechanism of NE differentiation induced by BMP-6 in vivo. 2) To investigate the mechanism of IL-6 induction by BMP-6 in macrophages. 3)To study the efficacy of dorsomorphin, a small molecule inhibitor of BMP signaling, on NE differentiation of prostate cancer in vivo. To date, aims 1 and 2 have been completed while aim 3 is currently progressing. When BMP-6 overexpressing prostate cancer cell line Tramp-BMP6 was injected subcutaneously into IL-6 knockout (KO) and conditional macrophage-null mice, neuroendocrine differentiation was no longer observed. Mechanistically, series of studies including shRNA knockdowns and immunoprecipitation assays have confirmed that Smad5 and GAT4 interact to induce IL-6 expression in macrophages. Currently, these finding are being confirmed in vivo using a small molecule inhibitor of BMP-6, dorsomorphine.					
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## INTRODUCTION

Prostate cancer is the most commonly diagnosed and the second leading cause of cancer deaths in the US (Jemal et al., 2009). Although surgery and radiation are quite effective for an organ-confined disease, the outlook for patients with a metastatic prostate cancer is still bleak due to the inevitable emergence of hormone refractory prostate cancer (HRPC). Since neuroendocrine (NE) cells are androgen receptor negative and frequently found in HRPC (Abrahamsson et al., 1998), understanding the biology of NE cells in prostate cancer may uncover new targets of intervention in patients with HRPC. In this regard, we have observed that the NE cells in human prostate cancer tissues were frequently positive for BMP-6 and co-localized with tumor associated macrophages (TAMs). In tissue culture, co-culture of macrophages and prostate cancer cells led to the expression of NE markers by prostate cancer cells in the presence of BMP-6. A survey of potential macrophage-derived mediators of NE differentiation demonstrated a dramatic induction of interleukin-6 (IL-6) by BMP-6. In investigating the mechanism of IL-6 induction by BMP-6 in macrophages, we have unexpectedly observed that both the canonical BMP-signaling pathway mediated by Smads and the non-canonical Smad-independent pathway mediated by p38 are required. Based on these results, the present grant proposal was funded to further dissect the mechanism as well as the biology of the tumor-host interaction loop composed of BMP-6, TAMs, and IL-6 in the context of NE cells in prostate cancer. This is the progress report for year 2 of the grant.

## BODY

Based on the preliminary data, we have hypothesized that **tumor-derived BMP-6 induces TAMs to express IL-6 via a crosstalk between the Smad-dependent and the p38 pathway; IL-6 in turn drives NE differentiation of prostate cancer cells.** To test this hypothesis, 3 specific aims were proposed: 1) To investigate the mechanism of NE differentiation induced by BMP-6 in vivo; 2) To investigate the mechanism of IL-6 induction by BMP-6 in macrophages; 3) To study the efficacy of dorsomorphin, a small molecule inhibitor of BMP signaling, on NE

differentiation of prostate cancer in vivo. Aim1 and parts of aim 2 were completed in year 1 and the past year was spent on completing aim 2 and part of aim 3.

Previously, we have demonstrated that both Smad-dependent and non-Smad pathways are required for the induction of IL-6 by BMP-6 in macrophages. Specifically, a physical interaction between Smad1 and GATA4 was required for BMP's activation of IL-6 promoter in macrophages. During the past year, we have observed that within the Smad-dependent pathway Smad1 alone is not sufficient for the activation of IL-6 promoter by BMP-6; in addition to Smad1, Smad4 was required (Fig 1). More importantly, when Smad4 was knocked down with shRNA, Smad1 translocated to the nucleus (Fig 2). These observations suggest that Smad4 is also required for the transcriptional activation of IL-6 promoter by BMP-6. Concerning the role of Smad-independent pathway and GATA4, we have confirmed that the expression of dominant negative GATA4 blocks the induction of IL-6 expression by BMP-6 (Fig 3).

As for the cross-talk between Smad1 and GATA4, immunoprecipitation previously confirmed a physical interaction. To further support this observation, we have attempted EMSA (electrophoretic mobility shift assay) as proposed in the task 2c of aim 2. But using the proposed antibodies against Smad1 and GATA4, we were not successful in demonstrating the supershifting of IL-6 promoter upon BMP-6 activation. Based on the results of the Smad4 overexpression study, we now propose that Smad4 is another critical player in BMP-6 induced activation of IL-6 promoter. Indeed, it is possible that Smad4 may be the critical link that connects Smad1/GATA4 to IL-6 promoter. We have recently initiated another EMSA experiment to test this hypothesis.

Simultaneously, we have begun investigating the effect of dorsomorphin, a small molecule inhibitor of BMP-6, on neuroendocrine differentiation of prostate cancer. To this end, a murine prostate cancer cell line with tetracycline-inducible expression of BMP-6 was used. This cell line, designated Tramp C2-BMP6, was injected into 40 C57BL/6 mice. The mice were divided into 4 groups as follows:

Cell Line	Tramp C2-BMP6 (n=40)			
Doxycycline	No (n=20)		Yes (n=20)	
Dorsomorphin	No (n=10)	Yes (n=10)	No (n=10)	Yes (n=10)

The tissues will be harvested and analyzed for NE differentiation in 2-4 weeks.

## KEY RESEARCH ACCOMPLISHMENTS

1. Completion of task 2b and progress on task 2c.
2. Initiation of animal studies examining dorsomorphine in NE differentiation of prostate cancer cells.

## REPORTABLE OUTCOMES

1. Published 1 manuscript.

Geun Taek Lee, Seok Joo Kwon, Jae-Ho Lee, Seong Soo Jeon, Kee Taek Jang, Han Yong Choi, Hyun Moo Lee, Wun Jae Kim, Seong Jin Kim, and Isaac Yi Kim. Induction of IL-6 expression by BMP-6 in macrophages requires both Smad and p38 signaling pathways. Journal of Biological Chemistry 285:39401-8, 2010.

## CONCLUSION

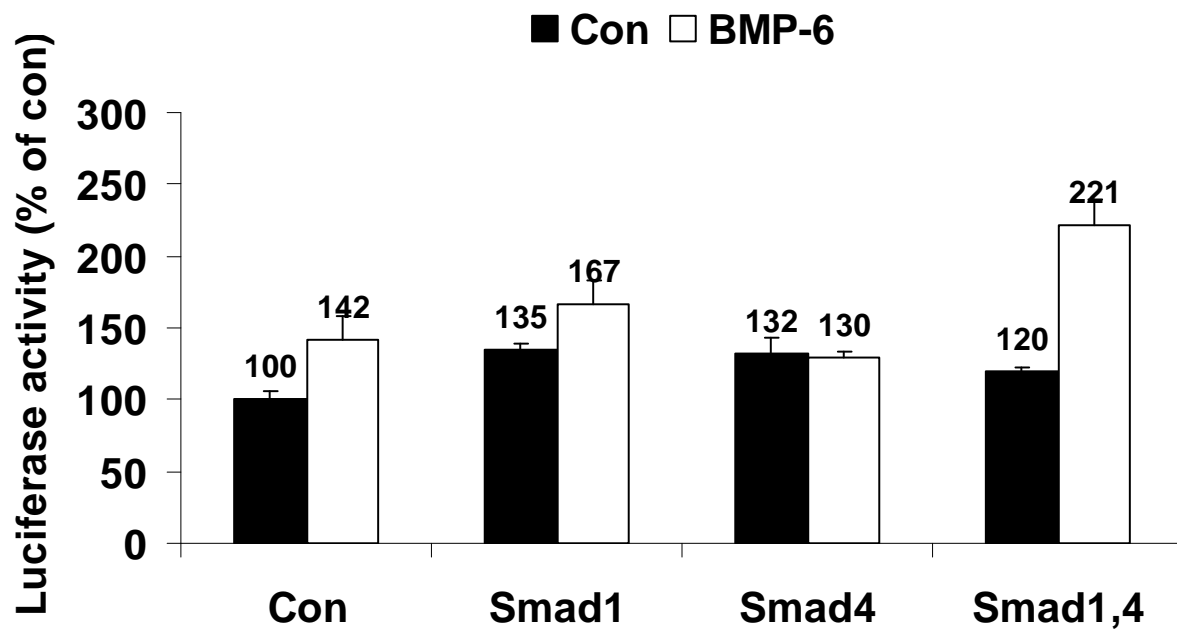
During the second year of funding, we have demonstrated that BMP-6 induces IL-6 expression in macrophages via Smad1/Smad4/GATA4 interaction. The third year of funding will focus on completing the preclinical studies using a small molecule inhibitor of BMP signaling to determine whether neuroendocrine differentiation of prostate cancer cells is reversible.

## REFERENCES

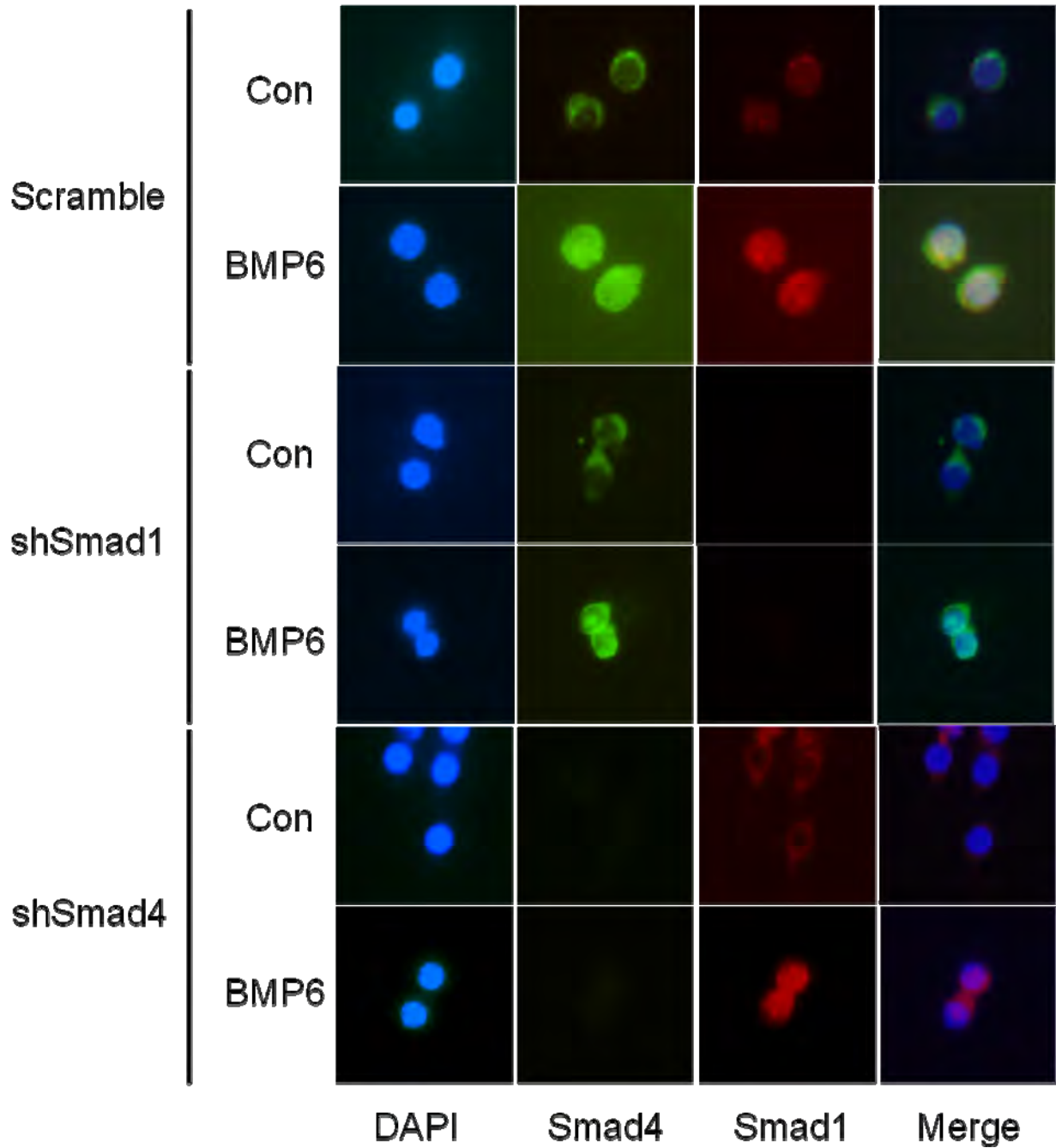
**Abrahamsson, P.A., Cockett, A.T., and di Sant'Agnese, P.A. (1998). Prognostic significance of neuroendocrine differentiation in clinically localized prostatic carcinoma. Prostate Suppl 8, 37-42.**

**Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., and Thun, M.J. (2009). Cancer statistics, 2009. CA Cancer J Clin 59, 225-249.**

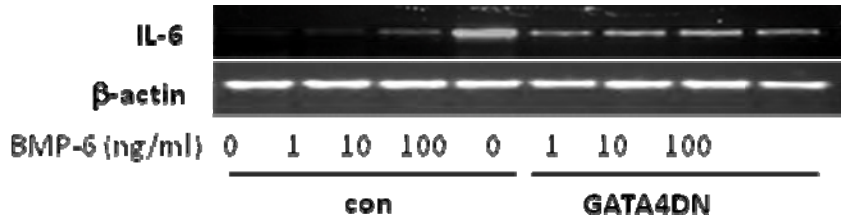
## Appendix



**Figure 1.** Smad1 and 4 were overexpressed in RAW 264.7 cells. When these cells were treated with BMP-6 100 ng/ml, only the simultaneous expression of both Smad1 and 4 increased BMP-6-induced IL-6 promoter activity as indicated by luciferase activity.



**Figure 2.** To determine the effect of Smad4 on nuclear translocation of Smad1, shRNA was used to knock-down Smad4. The results demonstrated that the knock-down of Smad4 had no significant effect on nuclear translocation of Smad1.



**Figure 3.** RAW 264.7 was transfected with dominant negative GATA4 (GATA4DN) and the effect on IL-6 expression was measured using RT-PCR. Following transfection with GATA4DN, baseline induction of IL-6 was again observed, suggesting that the transfection process itself can influence IL-6 expression. When treated with BMP-6, induction of IL-6 was no longer observed in cells expressing GATA4DN.